Short Report

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Endothelin-3 Gene Mutations in Isolated and Syndromic Hirschsprung Disease

Abstract

Hirschsprung disease (HSCR, aganglionic megacolon) is a frequent congenital malformation regarded as a multigenic neurocristopathy. Four susceptibility genes have recently been identified in HSCR, namely the RET proto-oncogene, the glial cell line-derived neurotrophic factor (GDNF), the endothelin B receptor (EDNRB) and the endothelin-3 genes (EDN3). Homozygosity for EDN3 mutations has been previously shown to cause the Shah-Waardenburg syndrome, a combination of HSCR with features of the Waardenburg syndrome. Here, we report on heterozygous EDN3 missense mutations in isolated HSCR. The present data give further support to the role of the endothelin-signaling pathway in the development of neural crest-derived enteric neurons. They also suggest the possibility that either recessive or weakly penetrant dominant alleles could occur at the EDN3 locus, depending on the nature of the mutation.

Introduction

Hirschsprung disease (HSCR, aganglionic megacolon) is a common congenital malformation (1/5,000 live births) characterized by the absence of intrinsic ganglion cells in the myenteric and submucosal plexuses along variable lengths of the gastrointestinal tract [1]. Since enteric neurons are derived from the vagal neural crest, HSCR is regarded as a neurocristopathy. The disease results in intestinal obstruction in neonates and severe constipation in infants and adults [2, 3]. Although more than 80% of HSCR cases are sporadic, several pedigrees have been described and a body of clinical, genetic and molecular data support the genetic heterogeneity of HSCR. In 1994, the RET proto-oncogene on chromosome 10 has been recognized as a major disease-causing gene in HSCR [4–7]. Yet, RET mutations only account for 50 and 15–20% of familial and sporadic cases of HSCR, respectively [8]. Recently, homozygous mutations of the endothelin-3 (EDN3) gene on chromosome 20 have been identified in consanguineous HSCR families harboring other malformations of neural crest-derived cells, namely pigmentary anomalies and deafness [Shah-Waardenburg syndrome, 9, 10]. However, the question of whether EDN3 muta-

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Table 1. *EDN3* gene mutations in patients with isolated HSCR or WS-HSCR association

Ref. No.	Exon	Familial or sporadic[1]	Phenotype	Nucleotide change	Protein change	Mutation	Origin of mutation[2]
9	2	S	WS-HSCR	delGC→T homozygous	truncation mutation	262 GC→T	F, M
10	3	F	WS-HSCR	G→T homozygous	TGC→TTC Cys→Phe	C159F	F, M
This report	2	F	WS-HSCR	G→T homozygous	GAG→TAG Glu→Stop	E55X	F, M
This report	1	S	HSCR	G→A heterozygous	GCA→ACA Ala→Thr	A17T	M
This report	5	S	HSCR	G→A heterozygous	GCT→ACT Ala→Thr	A224T	M

The first nucleotide of the transduction initiation codon is numbered as +1.

[1] F = Familial; S = sporadic.

[2] F = Father; M = mother.

tions could also account for isolated HSCR remained unanswered. Here, we report a homozygous *EDN3* mutation in a family with the Shah-Waardenburg syndrome (WS) and two heterozygous missense *EDN3* mutations in patients with isolated HSCR. These data further support the role of the endothelin signaling pathway in determining HSCR and raise the question of the mechanism(s) through which *EDN3* gene mutations could be deleterious.
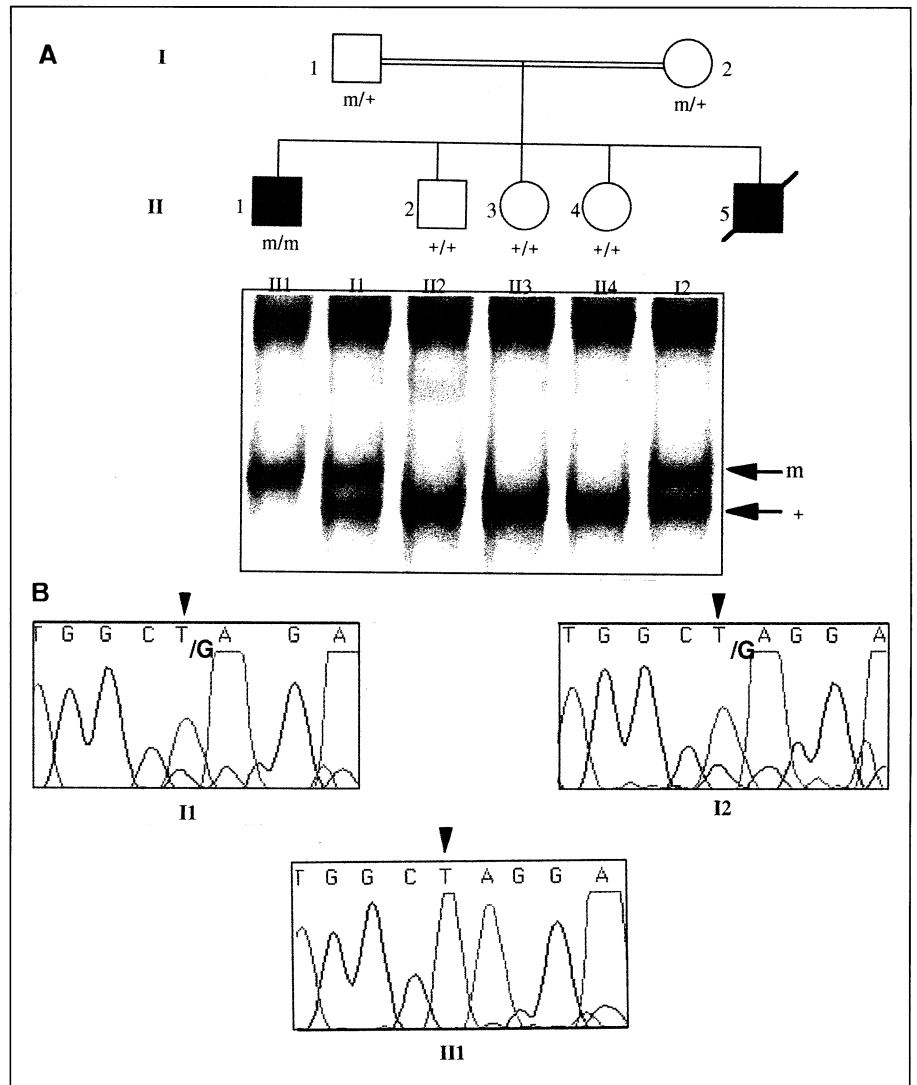
Patients and Methods

A total of 174 probands with isolated HSCR (59 families, 117 sporadic cases), and 6 patients with the WS-HSCR association (2 families, including 1 initially described by Van Camp et al. [11], and 4 sporadic cases) were screened for mutations in the coding sequence of the *EDN3* gene. Histopathological criteria for HSCR were (1) absence of enteric plexuses with histological evaluation of the aganglionic tract, and (2) increased acetylcholinesterase histochemical staining in nerve fibers. Long- and short-segment HSCR were defined according to the length of the aganglionic segment compared to the rectosigmoidal flexure. DNA was isolated from peripheral blood lymphocytes by standard methods. For PCR amplification of the *EDN3* coding sequence, 6 oligonucleotide primer pairs, encompassing the 5 exons and flanking intronic sequences, were used. Oligonucleotide sequences were as follows: (forward/reverse, 5'-3') exon 1, CAA GCG GCC GTC CTC CTG GTC CGG T/CTT CTC CGC GCC TCG GTC C (180 bp); exon 2A, CCC TCC TCA GGT GTT TGG G/TCG GCC GCC TGC TCC TGC (239 bp); exon 2B, TGG CGA GGA GAC TGT GGC T/GAA TGA GCA GAT GTG GGC G (218 bp); exon 3, GCT CAC CTA ACA TTA CCC/CCG AGG GTT GAT GCA TAT (259 bp); exon 4, GGT GGG GAA GAG GAA GTC A/CCC GAA GGA ATG ACA CGT C (200 bp); exon 5, TCA CAG AAC TAC AGA GCT AC/ACT GTG TGT GAG CAA TGA CA. Thirty-two amplification cycles were performed with the following annealing temperatures: 65°C, 60°C (6% formamide), 60°C, 52°C, 65°C and 55°C for exons 1, 2A, 2B, 3, 4, 5, respectively. For single-strand conformation polymorphism analysis (SSCP), PCR products were heated (10 min at 94°C), loaded onto a Hydrolink MDE gel (Bioprobe), then electrophoresed and autoradiographed for 36 h. In case of SSCP mobility shifts, amplified genomic DNA was sequenced on both strands by the fluorometric method (Dye Deoxy Terminator Cycle Sequencing kit, Applied Biosystems). For each DNA variant, 100 normal controls (200 chromosomes) were screened in addition to the unaffected family members in order to distinguish nonpathological base substitutions from disease-causing mutations.

Results

Our strategy was to analyze the coding sequence of the *EDN3* gene for mutations by using a combination of SSCP analysis and direct DNA sequencing. We found abnormal SSCP migration patterns in 3 unrelated HSCR probands (table 1). A homozygous G to T transversion at the first nucleotide of codon 55 was detected in exon 2 of the *EDN3* gene in a patient with the WS-HSCR association (fig. 1). This base substitution is a nonsense mutation (E55X) located upstream from the mature *EDN3* sequence, which presumably results in the complete absence of EDN3 in the proband. Both E55X mutant alleles were inherited from the consanguineous parents who were first cousins (f = 1/16) and did not show any clinical sign of WS and HSCR. None of the sibs carried the mutation, but an older brother had died with long segment

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Fig. 1. A Pedigree of the W1 family and SSCP patterns of the *EDN3* exon 2. + = Normal band; m = mutant band. **B** Direct DNA sequence of part of exon 2 of the *EDN3* gene showing the E55X mutation. The G to T transversion is indicated by an arrow.
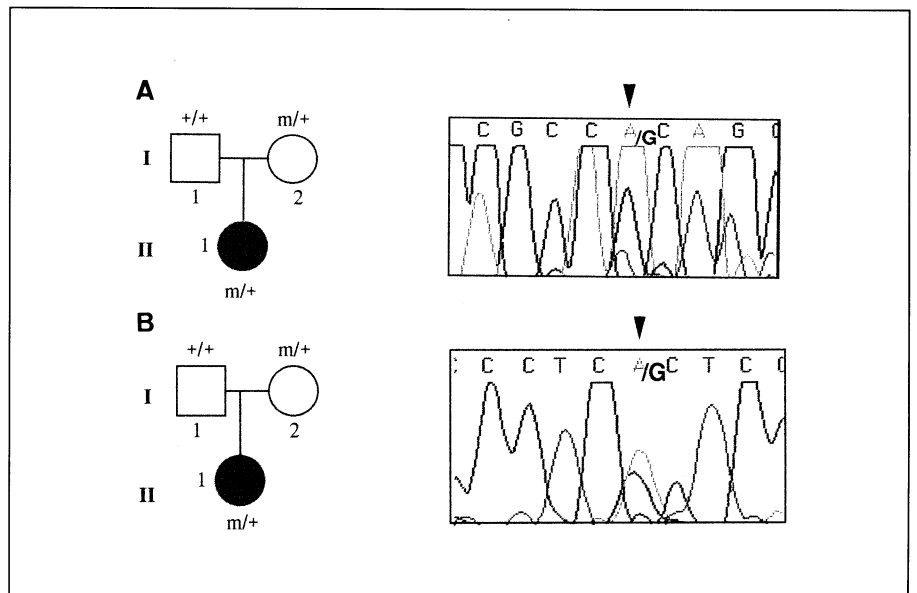
Fig. 2. A Pedigree of the S120 family. Direct DNA sequence of exon 1 of the *EDN3* gene showing the A17T mutation. The G to A transversion is indicated by an arrow. **B** Pedigree of the S65 family. Direct DNA sequence of exon 5 of the *EDN3* gene showing the A224T mutation. The G to A transition is indicated by an arrow. + = Normal allele; m = mutant allele.

HSCR and WS features [11] (fig. 1A). In 3 sporadic cases with WS-HSCR, no mutation was found in the open reading frame of *EDN3*.

In 2 patients with isolated and sporadic HSCR, direct DNA sequence showed heterozygosity for a G to A transition in exons 1 and 5 of the *EDN3* gene, respectively, at codons 17 and 224 (fig. 2). Both missense mutations (A17T and A224T) are predicted to result in the replacement of an alanine by a threonine residue in the preproendothelin. In particular, the A17T mutation is located in the peptide signal sequence. In both cases, the mutation was inherited from the asymptomatic mother.

Each of the three mutations was absent in 100 unrelated healthy individuals (200 chromosomes, $p < 0.01$). Finally, the rest of the *EDN3* coding sequence was normal, and no abnormal SSCP pattern was detected in the coding sequences of the *RET, GDNF* and *EDNRB* genes (data not shown).

Discussion

The *EDN3* gene encodes a large inactive preproendothelin-3 precursor which yields a biologically active 21-amino acid peptide after proteolytic cleavages at furin and endothelin-converting enzyme 1 cleavage sites, respectively. EDN3 belongs to a superfamily that also includes EDN1 and EDN2. Endothelins exert their biological effects through the heterotrimeric G protein-coupled heptahelical receptors A (EDNRA) and B (EDNRB) [12, 13]. Recently, transgenic mice with a homozygous knock-out mutation of *Edn3* were shown to have megacolon and coat color spotting (mutant *lethal spotted s'* [14, 15]. This gives support to the view that Edn3 is involved in the development of the enteric nervous system as previously shown for its receptor, Ednrb. Moreover, homozygous mutations of the human *EDN3* gene were shown to be associated with the pleiotropic features observed in the WS-HSCR syndrome in human [9, 10]. Here, we report the existence of a homozygous nonsense *EDN3* mutation (E55X) in another family with the WS-HSCR association. Since the E55X mutation is located upstream from the mature *EDN3* coding sequence, the nonsense mutation is predicted to result in the complete absence of the active form of the peptide in patient II1 (fig. 1). Both healthy parents were found to be heterozygous, suggesting that the E55X mutation might behave as a recessive allele.

However, the question of whether *EDN3* mutations could also account for isolated HSCR remained unanswered. Here we report on two heterozygous base substi-

tutions of the *EDN3* gene in a large series of nonsyndromic HSCR patients (174 probands). The missense mutations reported here occurred in conserved regions of the protein. In particular, the A17T mutation is located in the signal sequence of the preproendothelin that is critical for the correct addressing of the protein. Conversely, the A224T mutation lies in a region that is enzymatically spliced off during the processing of the preproendothelin. However, the substitution of a threonine for an alanine is a change from a nonpolar to a polar amino acid that might well affect the proper enzymatic cleavage of preproendothelin, a process in which the A17T mutation might also be involved. Thus, further in vitro functional studies are necessary to demonstrate the pathogenicity of the A17T and A224T mutations, although their absence in 200 control chromosomes ($p < 0.01$) argues against neutral polymorphisms which cannot be excluded, and suggests that they might be significant in the disease phenotype. Interestingly, both *EDN3* mutations reported here were consistently inherited from an unaffected mother carrier. This feature suggests a low penetrance of the trait and, eventually, the existence of one or more modifier loci in HSCR individuals carrying heterozygote *EDN3* mutations. Alternately, genomic imprinting of the *EDN3* locus with a preferentially expressed maternal allele could be discussed.

Thus, it is possible that the dosage of *EDN3* mutations could have various consequences on the resulting phenotype: heterozygosity would predispose to isolated HSCR with incomplete penetrance, while homozygosity would result in more complex neurocristopathies associating HSCR and WS features. Yet, it is worth remembering that mutations of either the *EDN3* [9, this report] or the *EDNRB* [16] genes were found in only 3/6 WS-HSCR probands examined so far in our series (2 familial forms including the one reported here, and 1 sporadic case, table 1), suggesting that other gene(s) could be involved in this complex neurocristopathy [9, 10, 16–18].

In conclusion, we propose here that the *EDN3* gene could be a rare susceptibility locus in nonsyndromic HSCR (2/174 probands in our series) as recently shown for another rare neurocristopathy, namely the congenital central hypoventilation syndrome [19]. Hitherto, four predisposing genes have been identified in isolated and syndromic HSCR, namely *RET, GDNF, EDNRB,* and *EDN3* [6, 7, 9, 10, 16–18, 20, 21]. This is in agreement with the prediction of genetic heterogeneity in HSCR. Moreover, the fact that *GDNF* mutations have been found so far in the context of mutations at the *RET* locus gives also support to the multigenic mode of inheritance

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of HSCR [20, 21]. Finally, while little is knownregarding the physiological role of EDN3, the involvement of EDN3 in isolated HSCR suggests that both RET and EDNRB signaling pathways are crucial for the development of the enteric nervous system. Further studies will hopefully address the question of whether mutations in genes encoding cytoplasmic proteins involved in signal transduction of the tyrosine kinase and the G-protein-coupled pathways could also be involved in the HSCR phenotype especially as cross talks between those two pathways have been demonstrated [21].

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